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Anthelmintic activity of *Zingiber roseum* rhizomes against *Pheretima posthuma*: In vitro and in silico approach

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ABSTRACT

Background : The goal of this study was to investigate the anthelmintic activity of a methanol extract of *Zingiber roseum* rhizome (ZRR) in an experimental model, followed by an in silico molecular docking study and an ADMET analysis.

Methods : A worm called *Pheretima posthuma* was used to test the anthelmintic activity. Then, molecular docking study was performed of identified compounds of *Z.roseum* rhizome from HPLC namelycatechin hydrate, epicatechin, trans-ferulic acid, rosmarinic acid,quercetin, myricetin, trans-cinnamic acid having maximum activity against β-TUBULIN enzyme by using CB-Dock protein-ligand docking server. Additionally, DruLiTo software was used to investigate drug-like predictions, and ADME/T profiles were examined using Swiss ADME Analysis and Molinspiration Cheminformatics software.

Results : ZRR contained alkaloids, flavonoids, tannins, and saponins, according to preliminary phytochemical analysis. On an adult worm *Pheretima posthuma*, ZRR exhibited dose-dependent and statistically significant anthelmintic activityThe best concentration of ZRR for anthelmintic activity on *Pheretima posthuma* was compared to the reference standard Albendazole (2.5 & 5.0 mg/ml).Our molecular docking study, on the other hand, reveals that the phenolic compounds (except trans-cinnamic acid) have strong binding affinities (ranging from -6.4 to -8.5 KCal/mol) to β-tubulin.These phyto-compounds from natural sources could be used to develop a reliable medication or to aid in lead identification using modern techniques.

Conclusion : The current study's findings confirmed *Zingiber roseum* rhizome extract's potential anthelmintic activity, and all compounds (except trans-cinnamic acid) were found to be effective in computer-aided drug design models.

Keywords : *Zingiber roseum*, Tannins, Anthelmintic, Molecular docking, QSAR prediction

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I. INTRODUCTION

Helminth infections are among the most common infections in humans, afflicting a large portion of the global population. Although most helminth infections are limited to tropical areas, they pose a significant health risk and contribute to the prevalence of malnutrition, anemia, eosinophilia, and pneumonia. Parasitic diseases inflict heinous morbidity, primarily on the people who live in endemic areas [1]. The gastro-intestinal helminthes develops resistance to currently available anthelmintic drugs, posing a major problem in the treatment of helminthes diseases. Elective medicine with healing has been used in the treatment of various problems since the beginning of time [2]. According to the WHO, 80 percent of the general population in a few Asian countries relies on regular medication in their daily components of medical care[3]. Approximately 25% of recommended drugs contain plant-derived segments, and approximately 121 active substances are currently used in drug items [4].

Tubulin is a well-known anticancer and anthelmintic drug target. The study of tubulin inhibitors could lead to the development of new antihelminthic medications. Furthermore, tubulin is a major constituent of microtubules, and the inhibitors bind selectively to β -tubulin of nematodes, cestodes, and flukes, disrupting microtubule structure and function[5-7]. Microtubules are extremely powerful, ubiquitous cell organelles that play a variety of essential roles in all eukaryotes, including mitosis, motility, and transport. Many of these designs exist in a unique equilibrium in which the gathering and dismantling of the dissolvable subunits are adjusted. In such contexts, drug-tubulin collaboration causes a shift in this harmony, resulting in a total deficit of microtubules and free tubulin aggregation[8]. Microtubules are also involved in a variety of cellular processes. Microtubule destruction eventually results in the organism's death [7]. Albendazole causes degenerative changes in the worm's intestinal cells by binding to the colchicine-sensitive site of β-tubulin and preventing its polymerization assembly into microtubules[9]. Albendazole is used as a standard drug in our study due to its broad spectrum activity, and there are numerous research articles available that support our findings[10, 11].

The in silico molecular docking technique is used in drug design and discovery to predict the conformations of each ligand molecule at the active site; thus, a molecular docking study was conducted to predict the tubulin inhibitory activity, and the results are reported.

The common name for *Zingiber roseum* is 'JangliAdrak' [12]. It has a tuberous rhizome, an erect stem, red flowers, white seeds, and long leaves. *Z. roseum* has a 1.2-1.5 cm long ligule, dull red spikes, a long peduncle, red petals with white colosur bases, a lump in the rhizomes, an entire labellum with a light yellow margin, and small fruits up to 6cm long[13]. This plant, which is a member of the Zingiberaceae family, has been documented for its traditional uses in a variety of diseases, in addition to its use in spices and as a decorative item. This plant is found in South China, India, Bangladesh, and Myanmar. It has traditionally been used to treat coughs, fevers, skin diseases, liver infections, and indigestion[14-16]. Traditional healers in Bangladesh use *Zingiber roseum* rhizomes to treat wounds, asthma, peptic ulcers, and rheumatic disorders [17]. According to research, rhizome aqueous extracts have antimicrobial, anticancer, and anti-inflammatory properties [18].

The photochemistry of *Z.roseum* has been studied, and several physiologically active compounds have already been identified from HPLC analysis in previous studies, such as catechin hydrate, epicatechin, quercetin, myricetin, transferulic acid, rosmarinic acid, and trans-cinnamic acid, which may be responsible for different pharmacological activity. Despite the fact that there has been no report demonstrating the anthelmintic activity of *Z.roseum* rhizome. As a result,

the current study aims to assess the anthelmintic activity of *Z.roseum* rhizome in experimental and computer-aided models.

II. MATERIALS AND METHODS

Collection of plant material

On October 21, 2019, rhizomes of *Zingiber roseum*Rosc. were collected from the Chittagong University hill tract in Chittagong, Bangladesh. Dr. Shaikh Bokhtear Uddin, Professor of Botany at the University of Chittagong, authenticated the plant material. (Accession no. SBU11064)

Preparation of extract

This plant's rhizomes were dried in the shade and ground into a fine powder in a laboratory mill. The dry powder is extracted with methanol over a 15-day maceration period [19].The resulting extracts were dried using a rotary vacuum dryer after filtration through Whatman filter paper and stored in sealed glass vials in a refrigerator at 4°C until further analysis.

Phytochemical tests

According to the standard procedures outlined in Pharmacopoeia, qualitative phytochemical studies of methanol extract were conducted to determine the presence of various secondary metabolites responsible for therapeutic value [20, 21]. The methanolic extract of *Z.roseum* was subjected to preliminary phytochemical testing for the identification of chemical constituents in accordance with standard operating procedures[22-25].

Experimental Animals

P. posthuma earthworms were collected from a waterlogged area of soil at Noakhali Science and Technology University in Bangladesh. Anthelmintic activity was investigated using adult earthworms *Pheretima posthuma*.To remove all fecal matter, they were washed with regular saline. For all experimental protocols, earthworms 5-8 cm long and 0.2-0.3 cm wide were used[11].

In Vitro anthelminthic activity

The antihelmintic activity of ZRR was determined using the previously described method [20]. The samples were triturated with 0.5 percent tween 80 and distilled water to create suspensions. The resulting mixture was then stirred for 30 minutes. The resulting suspensions were diluted to make concentrations (2.5mg/ml and 5mg/ml). These suspensions were used to treat worms. Albendazole, the standard drug, was also used in suspension form. As a control, a suspension of distilled water and tween 80 (0.5 %) was used. The worms were divided into groups of five earthworms each. In different petridishes, 20 mL of samples, standard, and control were poured. Before using the earthworms, they were all washed in a standard saline solution. In each, five worms of the same type were placed. The time for paralysis was recorded when there was no movement observed except when the worms were vigorously shaken. After determining that the worms did not move when shaken vigorously or dipped in warm water (50°C), the time for death was recorded.

Docking study

Ligand preparation

ChemBio Office version 12.0, a chemically intelligent drawing interface freeware developed by Advanced Chemistry Department, was used to obtain the smiles formulas of the selective ligands. Chem 3D, which was used to generate 3D coordinates from smiles, is available in Molecular Network software packages. Again, using the same server's Converter, the 2D structures of the drugs were converted into PDB format (Figure 1), which is an acceptable format for any standard docking software. Finally, ligand molecules were created using the Discovery studio visualizer by adding hydrogen atoms.

Preparation of Protein

The crystallographic structure of the target β-tubulin (1SA0) was obtained from PBD (Protein Data Bank)[26]and saved in standard 3D coordinate format (Figure 2). Because the targets possessed a selective ligand, active site residue identification was performed. Active Site Explicit Preparation Hydrogen atoms that were missing in the PDB structure were

added using the Discovery studio visualizer software. Furthermore, an atom list of the molecules was created, which represents the numbers of all the atoms of the active site residues involved.

Energy Minimization

Chem3D pro software version 12.0 was used to minimize the energy of the selective ligands. The RMSD deviation of new energy levels was checked with its actual PDB structure.

Molecular Docking

Docking is a method for predicting the preferred orientation of one molecule to another when they are bound together to form a stable complex.CB-DOCK Cavity-detection guided docking was used[27], the proteins were refined by removing water molecules and adding polar hydrogens and kollmann charges. The binding affinities are compared to the docking score of the standard ligand Albendazole.

Visual Inspection

Every drug candidate's solution structures against the targets were visualized and inspected for goodness of fit and orientation within the active site. PYMOL and the Discovery studio visualizer were used to create this. Both amino acid conformation and contacts were manually tested as well.

PASS Computer Program

PASS is a computer-based program for predicting various types of physiological behavior for a variety of compounds containing phytoconstituents. A substance's estimated activity is predicted as probable activity (Pa) and probable inactivity (Pi). The substances that show Pa greater than Pi are the only ones that are considered viable for a specific medical operation [28-30].

Drug-likeness and ADMET analysis

DruLiTo open-source software was used to perform a drug-likeness investigation to see whether there was any cytotoxicity to humans in previous report [31]. Ligand's pharmacological significance is based on its bioavailability or pharmacogenicity, which is calculated based on certain physiochemical and structural characteristics. The DruLiTo software then evaluated all ligands for their pharmaceutical nature according to Lipinski's five rules [32].

The ADMET of ligands are their pharmacokinetic properties, which must be examined in order to determine their function within the body. During the previous study[31]Molinspirationcheminformatics software was used to examine the heritage of the ADMET ligands.

Statistical Analysis

All data was presented as the mean±SEM; data was analyzed using a one-way ANOVA followed by Tukeyposthoc tests. GraphPad Prism (Version 8) was used to conduct the analytical assessment. The significance of $P< 0.05$ was considered statistically significant.

RESULTS

Phytochemical screening of the extract

Phytochemical testing of methanolic extract of *Z.roseum* rhizome revealed a variety of phytoconstituents, which are mentioned in Table 1.

Anthelminthic activity

On *P. posthuma* worm, ZRR anthelmintic activity has been determined. The degree of anthelmintic activity was found to be directly proportional to the concentration of the extract, ranging from 2.5 to 5.0 mg/ml. At concentrations of 2.5 and 5.0 mg/ml, ZRR displayed significant paralysis times of 60.66±0.85 minutes, 49.66±0.88 minutes and significant death times of 210.33±0.85 minutes, 172.66± 0.57 minutes respectively where the standard drug, Albendazole showed a paralysis time at the concentration of 2.5 and 5.0 mg/ml at 50.66±0.78 minutes, 45.33 ± 0.33 minutes and death times of 180.66± 0.66 minutes, 158.0 ± 0.57 minutes respectively. Table 2& figure 3-4 shows the outcomes for all of the categories.

Computational Study

In the current study, the score for the docking was used to verify the potential binding affinity of substances responsible for anthelmintic activity. Selective seven ligands and albendazole were used to

obtain docking free binding affinities against 1SA0 (protein chain A of the -tubulin enzyme). Table 3 shows the results obtained by the CB-Dock. When compared to standard Albendazole, some ligands showed strong binding affinity. The free binding affinities of seven compounds that target specific receptors were compared to the free binding affinity of the antihelminthic drug Albendazole.The free binding affinity of -6.3 Kcal/mol with β-tubulin was present in Albendazole. The binding affinity of Quercetin, Myricetin, Catechin hydrate, Transferulic Acid, Epicatechin, Rosmarinic Acid was higher compared to standard drug.Table 4 provides the interaction between the amino acid residue of target proteins and atoms of selected ligands and the interaction poses produced in docking studies are shown in Figure 5&6.

Drug Likeness and ADMET analysis

An analysis of their physiochemical interaction with the target domain is a crucial step in determining the drug-like properties of a compound. In the QSAR study, potential biochemical interactions between the compounds selected are predicted. In order to determine its physicochemical interaction with a certain objective, the study assessed seven ligands selected for several QSAR descriptors under Lipinski's five rules in previous study[31]. In the discovery and development of new drugs, this helps us assess their potential as medicine candidates and finally finds a new leading compound for a particular goal. In addition to the selective ligand ADMET properties, they were also reported[31].

PASS Predictions for Anthelminthic Activity

Online PASS result was obtained for the biological activity spectrum of the recognized phytoconstituents. These forecasts were flexibly interpreted and applied in table 5.

III. DISCUSSION

Natural compounds obtained from plants have piqued interest as a potential source of new healing agents. Many scientifically active drugs are from all-natural elements that demonstrate the importance of all natural medicinal products for medicinal exploration. This view has been taken to examine the plant, *Z. roseum*, for the evaluation of the anthelmintic task using earthworms, as well as to evaluate the in silico-molecular docking test and ADME/T [33-35].

Parasitic infection is a serious concern in both humans and animals, contributing to a catastrophic and lifelong illness that can lead to death, as well as causing drug resistance in some cases[36]. To avoid helminth contamination, study should concentrate on all-natural drugs, such as medicinal plants with novel bio-active substances that have little or no unintended side effects, are widely available in industrialized countries, and are far more in line with human biology than traditional medicines[37, 38].Monitoring has been carried out in this examinations; the time is regarded as paralysis. In addition, the fatality of earthworms against albendazole is viewed as a methanol extract and traditional therapy. On the worm, albendazole causes a flaccid paralysis that leads to peristalsis, which causes the worm to be expelled[35].During the preliminary phytochemical examination the *Z. roseum* rhizome methanol extract related to the anthelmintic activity was mainly identified by several bioactive phytoconstituents such as alkaloids, flavonoids, saponins and tannins[34].

ZRR was discovered to have significant anthelmintic potential in a dose-dependent manner in the current study. This behaviour may be caused by the existence of bioactive phytoconstituents including alkaloids, tannins, flavonoids, and saponins. Certain phytoconstituents, such as alkaloids and tannins, can induce significant anthelmintic activity[39]. Alkaloids can cause paralysis by binding to the central nervous system (CNS), while tannins and polyphenols selectively bind to free proteins in the gastrointestinal tract (GI tract) and eventually kill. Saponins, on the other hand, are anthelmintic due to their tendency to permeate membranes [34, 38]. The antihelmintic activity of the ZRR may be due to a particular

compound or to a mixture of these phytochemicals. The effective dosage for our sample was calculated to be 5.0 mg/ml ZRR, with paralysis and death periods of 49.66 and 172.66 minutes, respectively. As opposed to Albendazole, it took longer to paralyze and eliminate the remaining concentration. At either of the doses examined, the worms became paralyzed and died. The amount of time it took to paralyze the worms was inversely proportional to the amount of force collected. Methanol extract obviously has a significant anthelmintic effect, according to studies. The observations above support the use of an anthelmintic, as indicated by folk medicine.

We also assessed the molecular docking of certain substances in order to demonstrate the coordination between compounds and protein at molecular level, so that the behavior of molecules in the connection of targeted proteins is seen and the biochemical mechanism of the anthelmintic action can be explained.Catechin hydrate (-8.2 kcal/mol), epicatechin (-8.1 kcal/mol), trans-ferulic acid (-6.4 kcal/mol), rosmarinic acid (-7.6 kcal/mol), quercetin (-8.3 kcal/mol), and myricetin (-8.5 kcal/mol) all showed significant docking scores that were comparable to those of the reference drug, albendazole (-6.3 kcal/mol).Transcinnamic acid (-5.6 kcal/mol) has the lowest docking score compared to the standard drug.The docking analysis clearly reveals that these compounds have the potential to be a successful choice for a new anthelmintic agent.

Myricetin has the strongest correlation with β-Tubulin (1SA0) protein complexes. The complex of β-tubulin and myricetin formed four hydrogen bonds, namely ILE A:171 (2.47), TYR A:224 (2.25), GLU A:183 (2.02), ASP A:69 (3.08), and one amino acid involved in hydrophobic interactions PRO A:173 (3.51) and GLU A:183(OE2) (3.95) electrostatic interactions are facilitated by the moiety. Albendazole, on the other hand, forms three hydrogen bonds with the amino acid residues, namely SER A:140 (2.95), THR A:145 (3.04), GLY A:146 (3.15), four hydrophobic interactions, namely ALA A:12 (3.90), ALA A:99 (4.20), ILE A:171 (3.99), TYR A:224 (5.29), and one electrostatic interaction named ASN A:206 (3.29).

The key amino acids involved in hydrogen bonding include GLN A: 11, which interact with catechin hydrate, transferulic acid, and rosmarinic acid in a usual manner. Furthermore, TYR A: 224 interacts with epcatechin, rosmarinic acid, quercetin, and myricetin. Except for quercetin and myricetin, all other ligands demonstrated excellent hydrophobic interactions with the amino acid ALA A: 12. Interestingly, GLU A: 183 have been implicated in both hydrogen bonding and electrostatic interactions. As a result, except for trans-cinnamic acid, all of the separated constituents exhibited the maximum binding affinity in contrast to the conventional medication.Several amino acid deposits in each ligand-protein connection regulate electrostatic, hydrophobic, and hydrophilic communications, according to our findings.

IV. CONCLUSION

According to the findings, the methanolic extract of *Z. roseum* rhizomes can play a significant role in anthelmintic action. Besides, in silico investigation revealed that, when compared to albendazole, all selective ligands except trans-cinnamic acid have the best binding effects. This finding strongly implies that the rhizomes of *Z. roseum* must contain lead compounds, which might have significant implication for developing new antihelmintic medications to eradicate helminthiasis worldwide.

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Figures and Tables

Table 1. Phytochemical screening of methanolic extract of *Zingiber roesum* rhizome (ZRR)
"+": Present; "–": Absent

Phytochemicals	Method	Methanolic extracts
Alkaloids	Wagner test Hager's test	+
Flavonoids	Lead acetate test Zn. hydrochloride test	+
Phenols	Ferric chloride test	+
Tannins	Ferric chloride test Vanillin-HCl test	+
Terpinoids	Salkowski test	+
Saponins	Foaming test	+
Glycosides	Keller-Kiliani test	_
Carbohydrates	Molish test	_

"+": Present; "–": Absent.

Table 2. Anthelminthic effect of methanol extract of *Zingiber roseum* rhizome
#Control sample was observed for 12 hours but has shown no paralysis.
The result was expressed as Mean ± SEM. (n=3)

Concentrations	Times In Minutes	
	Time taken for Paralysis	Time taken for Death
Sample 2.5mg/ml	60.66±0.85**	210.33±0.85****
Sample 5.0mg/ml	49.66±0.88**	172.66± 0.57***
Albendazole 2.5mg/ml	50.66±0.78**	180.66± 0.66****
Albendazole 5.0mg/ml	45.33 ± 0.33**	158.0 ± 0.57***

#Control sample was observed for 12 hours but has shown no paralysis.

The result was expressed as Mean ± SEM. (n=3).

****P <0.0001, ***P < 0.001, **P < 0.005, *P < 0.05 versus Standard (Albendazole).

****P <0.0001, ***P < 0.001, **P < 0.005, *P < 0.05 versus Standard (Albendazole).

Table 3. Binding affinities of isolated compounds and standard at the active site of β-Tubulin

Ligands	Highest to the lowest mode of conformation with corresponding RMS binding affinities in ΔG (Kcal/mol)				
Catechin hydrate	-8.2	-6.7	-6.6	-6.3	-5.3
Epicatechin	-8.1	-6.7	-6.6	-6.3	-5.8
Trans-ferulic acid	-6.4	-5.7	-5.6	-5.1	-5.0
Rosmarinic acid	-7.6	-6.9	-6.1	-5.9	-5.4
Quercetin	-8.3	-7.1	-7.0	-6.2	-5.4
Myricetin	-8.5	-7.2	7.0	-6.3	-5.7
Trans-cinnamic acid	-5.6	-5.6	-5.3	-4.6	-4.4
Albendazole	-6.3	-6.0	-5.7	-5.4	-5.1

Table 4. Interactions of β-Tubulin amino acid residues with ligands at receptor sites

Ligands	Binding Affinity, ΔG (Kcal/mol)	Amino acids involved and Distance (A°)		
		Hydrogen binding interactions	Hydrophobic interactions	Electrostatic interactions
Catechin hydrate	-8.2	SER A:178 (2.19), GLN A: 11 (2.48)	ALA A:12 (5.19)	--
Epicatechin	-8.1	GLY A:146 (3.07), TYR A:224 (3.12), VAL A:177 (2.21), ASN A:206 (2.58)	ALA A:12 (4.94)	GLU A:183 (4.67)
Trans-ferulic acid	-6.4	GLN A:11 (3.11), GLY A:144 (3.15), THR A: 145 (2.83), GLY A:146 (2.98)	PRO A:173(4.68), ALA A:12 (4.05)	--

Rosmarinic acid	-7.6	GLN A:11 (3.28), SER A:140 (2.85), TYR A:224 (2.97), SER A:178 (2.59), ASP A:69 (3.33), ASN A:206 (2.26)	ALA A:12 (4.78)	--
Quercetin	-8.3	VAL A:177 (2.81), TYR A:224 (2.16), GLU A:183 (2.06)	--	GLU A:183(OE2) (3.86)
Myricetin	-8.5	ILE A:171 (2.47), TYR A:224 (2.25), GLU A:183 (2.02), ASP A:69 (3.08)	PRO A:173 (3.51)	GLU A:183(OE2) (3.95)
Trans-cinnamic acid	-5.6	--	ALA :12 (3.40), TYR A:224 (3.83), GLN A:11 (4.83)	--
Albendazole	-6.3	SER A:140 (2.95), THR A:145 (3.04), GLY A:146 (3.15)	ALA A:12 (3.90), ALA A:99 (4.20), ILE A:171 (3.99), TYR A:224 (5.29)	ASN A:206 (3.29)

Table 5. PASS analysis of Zerumbone from *Z. roseum*

Main predicted activity by PASS online	**Name**	**Pass-online activity**	
		Pa	**Pi**
Anthelmintic activity	Catechin hydrate	0.400	0.042
	Epicatechin	0.400	0.042
	Trans ferulic acid	0.583	0.008
	Rosmarinic acid	0.230	0.068
	Quercetin	0.362	0,060
	Myricetin	0.346	0.070
	Trans cinnamic acid	0.673	0.004

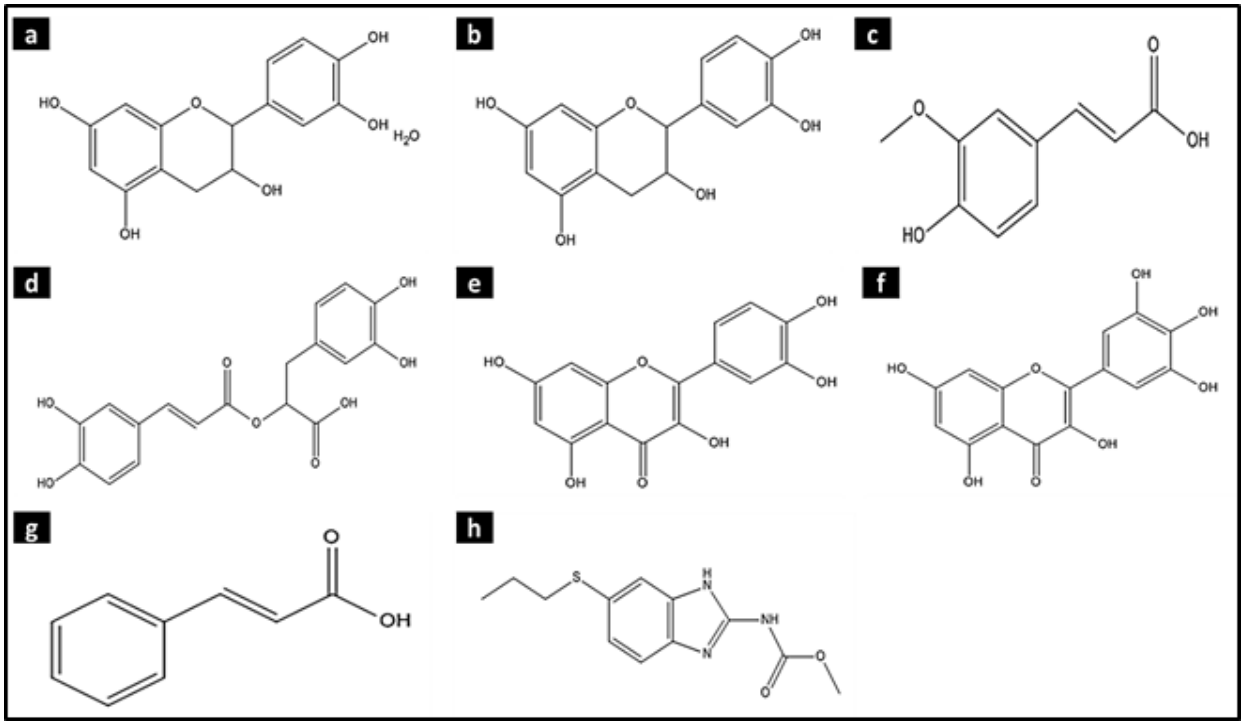


Figure1: The two-dimensional structures of in silico active ligands. (a) Catechin hydrate; (b) Epicatechin; (c) Trans-ferulic acid; (d) Rosmarinic acid; (e) Quercetin; (f) Myricetin; (g) trans-cinnamic acid; (h) Albendazole

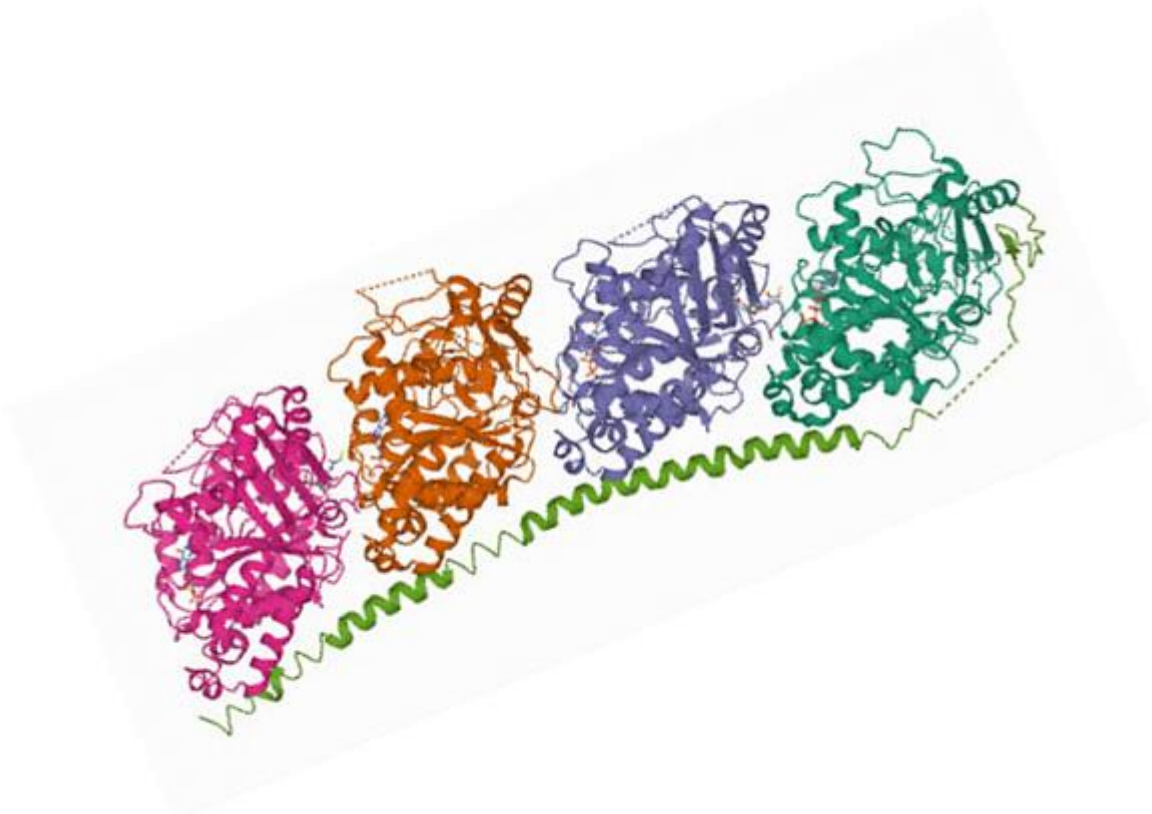


Figure 2: Three-dimensional structure of the molecular target, Tubulin-Colchicine (1SA0)



Figure 3 : Anthelminthic activity ZRR methanol extract and standard drug; (a) Normal control, (b) Sample 2.5 mg/ml, (c) Albendazole 5.0 mg/ml, (d) Sample 5.0 mg/ml, (e) Albendazole 5.0 mg/ml

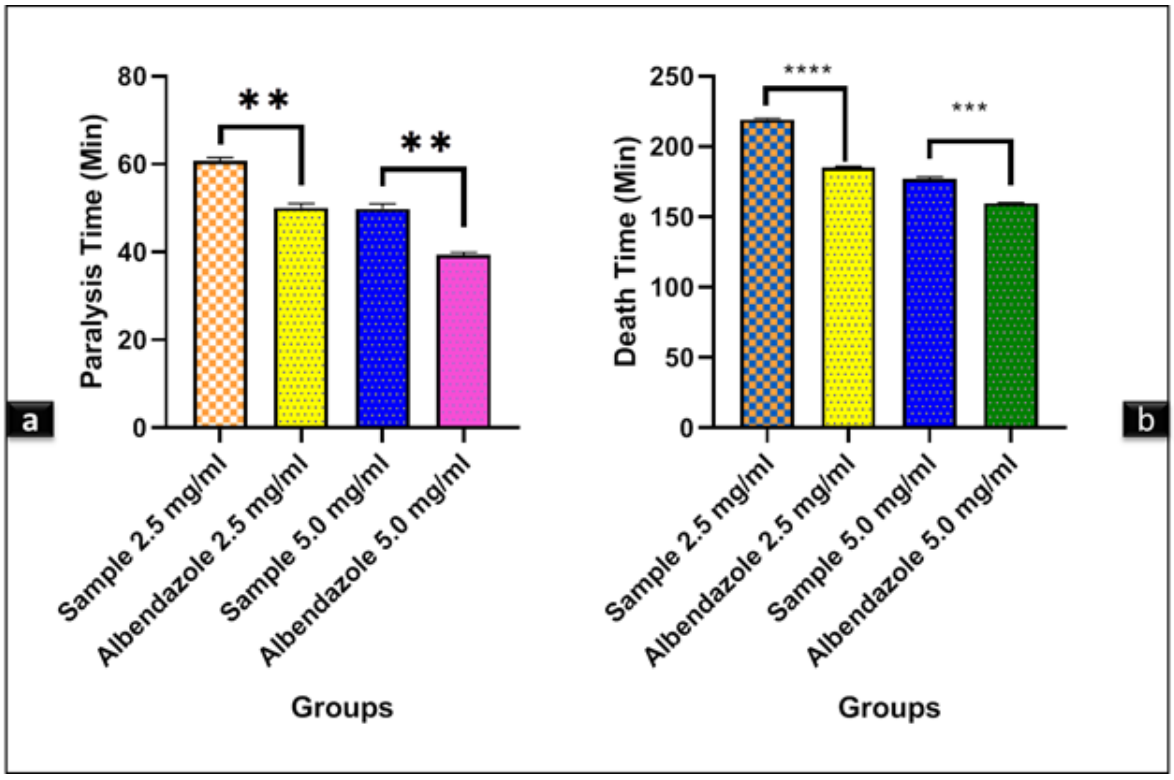


Figure 4: (a) Paralysis time for methanol extract of *Zingiber roseum* with standard albendazole. (b) Death time for methanol extract of *Zingiber roseum* with standard albendazole.

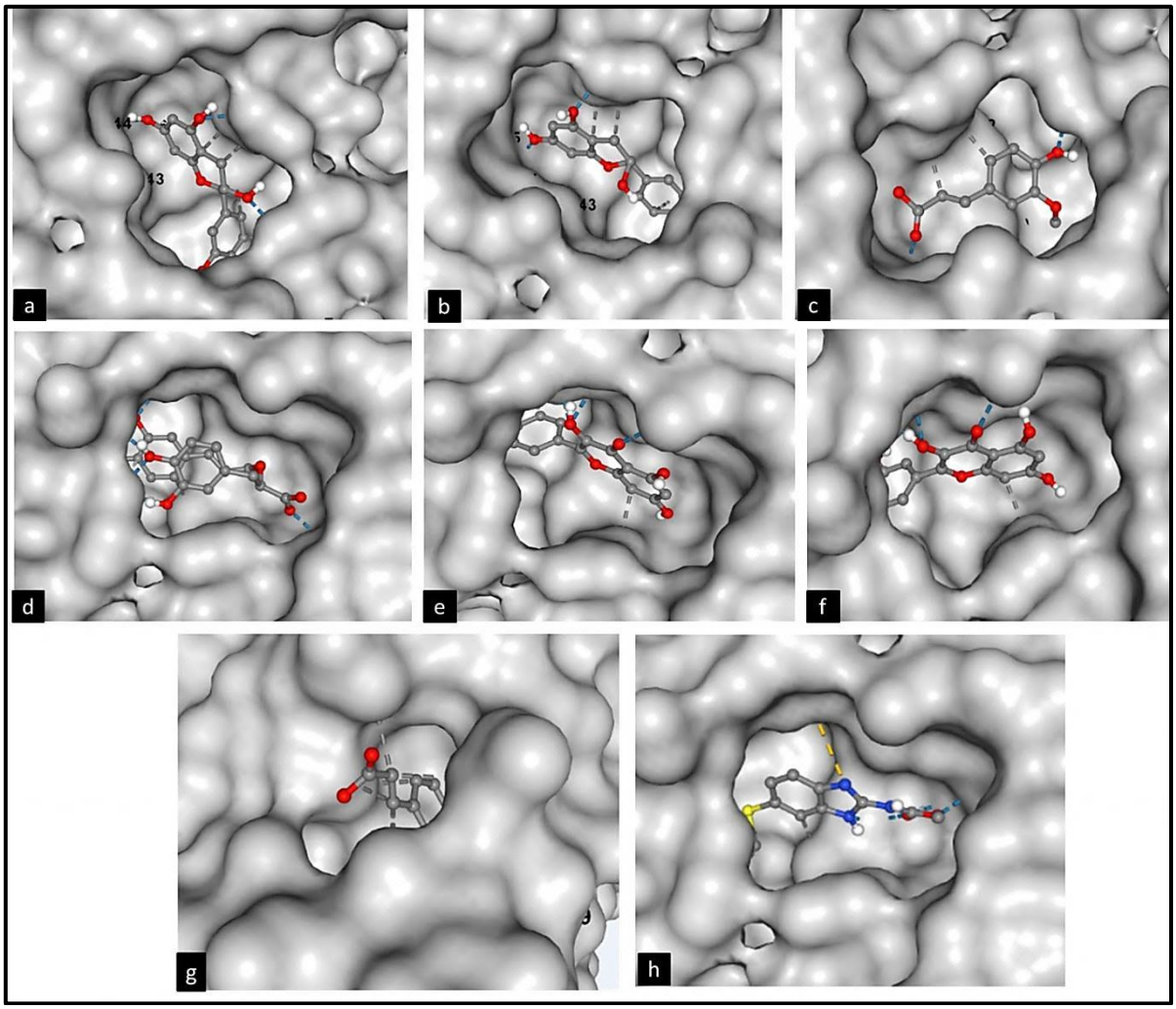


Figure 5: In silico docked complexes of ligands (Ball and Stick representation) with β-Tubulin. (a) Catechin hydrate; (b) Epicatechin; (c) Trans-ferulic acid; (d) Rosmarinic acid; (e) Quercetin; (f) Myricetin; (g) trans-cinnamic acid; (h) Albendazole

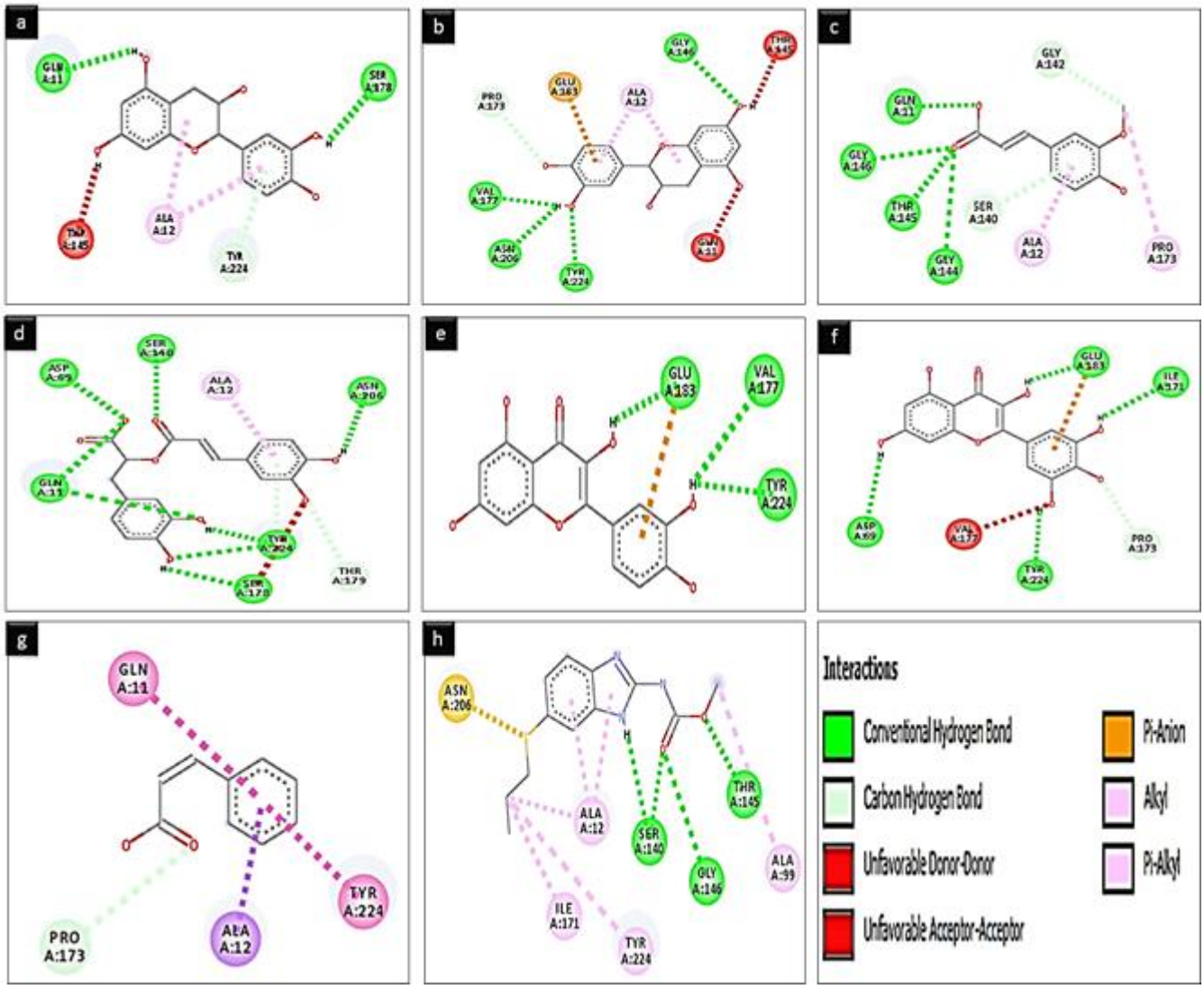


Figure 6: Possible 2D interactions of (a) Catechin hydrate and β-tubulin, (b) Epicatechin and β-tubulin, (c) Transferulic acid and β-tubulin, (d) Rosmarinic acid and β-tubulin, (e) Quercetin and β-tubulin, (f) Myricetin and β-tubulin, (g) Trans-cinnamic acid and β-tubulin, (h) Albendazole and β-tubulin.